Pentose Metabolism in *Rhizobium leguminosarum* MNF300 and in Cowpea *Rhizobium* NGR234

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L-Arabinose is broken down by *Rhizobium leguminosarum* MNF300 via 2-oxoglutarate semialdehyde. Enzyme activities in cells grown on succinate, mannitol or arabinose indicated much greater modulation of arabinonate dehydratase, 2-keto-3-deoxyarabinonate dehydratase and 2-oxoglutarate semialdehyde dehydrogenase than of arabinose dehydrogenase or of arabinono- γ -lactonase. In cowpea *Rhizobium* NGR234, all the enzymes of L-arabinose metabolism except L-arabinono- γ -lactonase were inducible. Assays for such enzymes in snake bean bacteroids indicated that L-arabinose did not reach the bacteroids in large quantities. The Tn5-induced mutant MNF3045 of *R. leguminosarum* was unable to grow on L-arabinose and accumulated L-arabinono- γ -lactone and L-arabinonate. Product accumulation and enzyme assays suggested that this mutant was defective in L-arabinonate dehydratase. It nodulated peas and the nodules fixed N₂, indicating that the supply of L-arabinose is not essential for bacteroid function. Another Tn5-induced mutant of *R. leguminosarum*, MNF3041, lacked ribokinase and was unable to grow on D-ribose; this mutant was also able to nodulate peas and fix N₂.

INTRODUCTION

Rhizobia can efficiently utilize a variety of pentoses for growth. It is therefore possible that the supply of such compounds by host legumes may be important to nodule formation or function. One approach to the identification of compounds which are important to symbiotic N₂ fixation in the legume nodule has been the study of rhizobial mutants blocked in particular steps in catabolism. A mutant of *Rhizobium meliloti* unable to grow on L-arabinose was identified as defective in the synthesis of 2-oxoglutarate dehydrogenase (Duncan & Fraenkel, 1979); it continued, however, to degrade L-arabinose at the same rate as the wild-type strain. A similar Tn5-induced mutant also metabolized L-arabinose without growth, and either nodulated later than the wild-type or formed no nodules at all (Duncan, 1981). Mutants of *R. meliloti* which were unable to grow on ribose or on xylose were normal in both nodulation and N₂ fixation, and still possessed the capacity to degrade these pentoses (Duncan, 1981). While these data suggest that metabolic intermediates were accumulating, none was identified.

The routes of pentose utilization in rhizobia are not well defined. In *Bradyrhizobium japonicum*, L-arabinose appears to be degraded to L-2-keto-3-deoxyarabinonate (KDA), which is then cleaved to pyruvate and glycolaldehyde (Fig. 1) via an aldolase reaction (Pedrosa & Zancan, 1974). While pyruvate and glycolaldehyde were identified as products of 2-keto-3-deoxyarabinonate, the alternative route, dehydration to 2-oxoglutarate semialdehyde, was not explored. It was subsequently reported (Duncan, 1979) that *B. japonicum* 61A76 and cowpea *Bradyrhizobium* 32H1 not only possessed KDA aldolase but also 2-oxoglutarate semialdehyde dehydrogenase. The presence of this enzyme suggests that KDA dehydratase may also have

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Abbreviation: KDA, 2-keto-3-deoxyarabinonate.

Fig. 1. Pathways of catabolism of L-arabinose in rhizobia.

been present (Fig. 1). In the fast-growing '*R. trifolii*', '*R. phaseoli*' [now regarded as biovars *trifolii* and *phaseoli* of *R. leguminosarum*], *R. leguminosarum* and *R. meliloti*, KDA aldolase was detectable only in *R. meliloti*, at low activity, while the activities of 2-oxoglutarate semialdehyde dehydrogenase were high in all four organisms. This suggested that dehydration of KDA was the preferred route in these organisms, and that the fast- and slow-growing rhizobia were fundamentally different in their metabolism of L-arabinose (Duncan, 1979). Little information is available concerning the route of D-ribose catabolism in rhizobia. The operation of the pentose phosphate pathway in rhizobia is generally assumed (Stowers, 1985); the likely route of metabolism is phosphorylation to ribose 5-phosphate and isomerization and epimerization to ribulose and xylulose 5-phosphates. The characteristics of a ribose-negative mutant of *R. meliloti* described by Duncan (1981) suggest that this is not the only route since that mutant was defective in ribokinase but still caused ribose disappearance.

We have re-examined the route of L-arabinose catabolism in *R. leguminosarum* MNF300 and in cowpea *Rhizobium* NGR234 and determined that it follows the 2-oxoglutarate semialdehyde pathway. In addition, we have isolated mutants defective in the metabolism of either D-ribose or L-arabinose and examined their symbiotic properties.

METHODS

Organisms. *Rhizobium leguminosarum* MNF300 (strain 300 of Johnston & Beringer, 1975) is a prototrophic strain which effectively nodulates peas. Mutants derived from this strain are listed in Table 1. Tn5-induced mutants MNF3041 and MNF3045 were obtained by use of the suicide plasmid pJB4JI as described by Beringer *et al.* (1978). Strain MNF3400 is a spontaneous mutant which has acquired the ability to grow on arabinonate, and was isolated by plating MNF300 (10^8 cells) on minimal salts medium containing L-arabinonate (5 mM) as sole source of carbon.

Cowpea *Rhizobium* NGR234, a fast-growing strain nodulating snake bean [*Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc.], was obtained from Dr M. J. Trinick, CSIRO, Canberra, Australia.

Media. Solid media were either Y basal medium (Beringer, 1974) or minimal salts medium (Brown & Dilworth, 1975), supplemented with single carbon sources at 5 or 10 mM. Liquid media contained minimal salts (Brown & Dilworth, 1975) but with 0.3 mM-phosphate, 10 mM-ammonium chloride and 10 mM-carbon source (except for D-arabitol, D-ribitol and L-arabinonate, which were at 5 mM), and were buffered to pH 7.2 with 40 mM-HEPES. All strains were grown at 28 °C.

Transduction. Phage RL38 (Buchanan-Wollaston, 1979) was used to demonstrate cotransduction of Tn5 with the nutritional lesions of Tn5⁺ mutants as previously described (Arwas *et al.*, 1985). Strain MNF340045 was constructed by RL38-mediated transduction of the Tn5-linked mutation of MNF3045 to MNF3400.

Nodulation assay. Mutants of *R. leguminosarum* were tested for nodulation and N₂ fixation (C₂H₂ reduction) on peas (*Pisum sativum* cv. Greenfeast). A minimum of 60 single-nodule isolates was recovered from plants which had been inoculated with each mutant, and it was confirmed that the phenotype of these was identical to that of the inoculant strain.

Table 1. *Strains of R. leguminosarum and their properties*
Growth* on:

Strain	Derivation	Phenotype†	Growth* on:				
			D-Ribose	L-Arabinose	L-Arabinonate	D-Ribitol	D-Arabitol
MNF300	Wild-type		+	+	—	+	+
MNF3041	Tn5 ⁺ mutants of MNF300	Rib [—]	—	+	ND	+	+
MNF3045		Ara [—]	+	—	—	ND	ND
MNF3400	Spontaneous mutant of MNF300	Aup ⁺	ND	+	+	ND	ND
MNF340045	Transductant of MNF3400‡	Ara [—] Aup ⁺	ND	—	—	ND	ND

ND, Not done.

* +, Single colonies visible after 5 d incubation at 28 °C; —, no macroscopically visible colonies after 5 d incubation at 28 °C.

† Phenotypic symbols: Rib, ribose; Ara, arabinose; Aup, arabinonate uptake.

‡ MNF340045 was constructed by phage RL38-mediated transduction of the Tn5-linked Ara[—] mutation of MNF3045 to MNF3400.

Nodules containing *Rhizobium* NGR234 were grown on a dwarf variety of snake bean supplied by Arthur Yates & Co., Perth, W. Australia, as previously described (Saroso *et al.*, 1984).

Isolation of bacteroids. Bacteroids were isolated from nodules by a modification (McKay *et al.*, 1985) of the Percoll gradient method of Reibach *et al.* (1981).

Preparation of cell-free extracts. Cells were centrifuged at 6000 *g* for 10 min at 4 °C and the pellet was washed twice with minimal salts (Brown & Dilworth, 1975) and resuspended in 20 mM-HEPES (pH 7.6) containing 1 mM-dithioerythritol. Cell-free extracts were prepared using a French pressure cell as described by McKay *et al.* (1985).

Uptake assays. Uptake of D-[¹⁴C]ribose or L-[¹⁴C]arabinonate was measured after cells had been washed twice by centrifugation and resuspended in minimal salts at 28 °C, with unlabelled ribose or arabinonate at 0.1 mM; the procedure was otherwise that of Hudman & Glenn (1980).

Radioactive substrates. D[1-¹⁴C]Ribose (58 mCi mmol^{−1}; 2.15 GBq mmol^{−1}) was from Amersham, UK, and L-[U-¹⁴C]arabinose (210 mCi mmol^{−1}; 7.77 GBq mmol^{−1}) from CEA, France.

L-[U-¹⁴C]Arabinonate (0.77 mCi mmol^{−1}; 28.5 MBq mmol^{−1}) was synthesized as follows. Carrier L-arabinose (3 mg) and 20 µCi [¹⁴C]arabinose were dried *in vacuo* in an Eppendorf tube, and then dissolved in 0.02 ml water at 80 °C. After addition of 0.14 ml of a solution containing 0.114 g I₂ in 1.6 ml methanol, 0.27 ml of a solution containing 1 g KOH in 25 ml methanol was added, and the tube was held at 47 °C for 10 min and then left at 4 °C overnight to allow crystallization. The white solid was dissolved in 0.05 ml water, reprecipitated by adding 0.75 ml methanol, and redissolved in 4 ml water for determination of radioactivity and L-arabinonate concentration. Radiochemical purity was established by chromatography on paper using ethyl acetate/pyridine/water, as described below. After development, the chromatograms were cut into 8 mm strips, and the radioactivity of each strip was counted. All radioactivity coincided with L-arabinonate.

Enzyme assays. All HEPES buffers used were adjusted to the appropriate pH with NaOH, and all Tris buffers with HCl.

L-Arabinose dehydrogenase (L-arabinose:NADP⁺ 1-oxidoreductase, EC 1.1.1.46) was assayed spectrophotometrically at 340 nm in a mixture containing (in 1.0 ml final volume) 80 µmol Tris buffer (pH 8.5), 0.4 µmol NADP⁺ and 10 µmol L-arabinose. L-Arabinono-γ-lactonase (L-arabinono-1,4-lactone lactonohydrolase, EC 3.1.1.15) was measured by incubating (in a 2.0 ml final volume) cell-free extract, 100 µmol HEPES buffer (pH 7.6) and 8 µmol L-arabinono-γ-lactone. Samples were taken at 0, 10 and 20 min for lactone assay.

L-Arabinonate dehydratase (L-arabinonate hydro-lyase, EC 4.2.1.25) was assayed by following the disappearance of L-arabinonate from a mixture containing (in a final volume of 2.5 ml) 5 µmol L-arabinonate, 125 µmol HEPES buffer (pH 7.6), 20 µmol MgCl₂ and 5 µmol 2-mercaptoethanol. Samples (0.3 ml) withdrawn at 0, 15 and 30 min were heated (5 min at 100 °C) with 0.03 ml HCl/H₂O (1:2, v/v) and assayed for lactone content.

KDA dehydratase (2-dehydro-3-deoxy-L-arabinonate hydro-lyase, EC 4.2.1.43) was measured by monitoring the disappearance of KDA from a mixture (in 1.0 ml final volume) containing 100 µmol potassium phosphate buffer (pH 7.6), 2 µmol 2-mercaptoethanol and 1.4 µmol DL-KDA. Samples (0.02 ml) were taken at 0, 15 and 30 min into 0.025 ml 0.125 M-H₂SO₄ for measurement of KDA.

2-Oxoglutarate semialdehyde dehydrogenase (2,5-dioxopentanoate:NADP⁺ 5-oxidoreductase, EC 1.2.1.26) was measured spectrophotometrically at 340 nm in a mixture containing (in a final volume of 1.0 ml) 100 µmol potassium phosphate buffer (pH 7.6), 0.4 µmol NADP⁺ (NAD⁺ for *R. leguminosarum* MNF300 and MNF3045), 2 µmol KCN and 0.1 µmol 2-oxoglutarate semialdehyde.

KDA aldolase (2-dehydro-3-deoxy-L-pentionate glycolaldehyde-lyase, EC 4.1.2.18) was measured by formation of KDA in a mixture containing (in a final volume of 1.0 ml) 100 μ mol potassium phosphate buffer (pH 7.6), 10 μ mol MgCl_2 , 10 μ mol sodium pyruvate and 10 μ mol glycolaldehyde. Samples were taken at 0, 15 and 30 min into 0.025 ml 63 mM- H_2SO_4 .

Ribokinase (ATP:D-ribose 5-phosphotransferase, EC 2.7.1.15) was measured essentially as described by Duncan (1981), except that the final ribose concentration was 10 mM and the buffer was 50 mM-HEPES (pH 8.2). Ribose-5-phosphate isomerase (D-ribose-5-phosphate ketol-isomerase, EC 5.3.1.6), ribulose-phosphate 3-epimerase (D-ribulose-5-phosphate 3-epimerase, EC 5.1.3.1) and transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycolaldehydetransferase, EC 2.2.1.1) were assayed in 50 mM-HEPES buffer (pH 7.5) by the methods of Novello & McLean (1968).

Reagents. Arabinono- γ -lactone solutions were prepared by heating L-arabinonate in 0.1 M-HCl (5 min at 100 °C) followed by careful partial neutralization with dilute NaOH before use. Potassium L-arabinonate and potassium D-ribonate were synthesized from L-arabinose and D-ribose, respectively, by the method of Moore & Link (1940), DL-KDA by the methods of Stoolmiller & Abeles (1966) and Stoolmiller (1975), and 2-oxoglutarate semialdehyde by the method of Adams (1971). Some 2-oxoglutarate semialdehyde was the generous gift of Dr Y. F. Chang, Department of Microbiology, University of Maryland, USA.

Assay of accumulating compounds in culture filtrates of arabinose mutants. HEPES at the concentrations used in the buffered growth media interfered with arabinono- γ -lactone or L-arabinonate assays by the method of Hestrin (1949). Accordingly, supernatants of cultures of mutants MNF3045 and 340045 were adjusted to pH 5.0 with HCl, and 20 ml samples passed through columns (6 cm $\times$ 6 mm diam.) of Dowex-50W (100–200 mesh, H^+ form) to remove HEPES. Control experiments indicated no losses of arabinono- γ -lactone or arabinonate in this procedure.

Identification of products from L-arabinose. The products of L-arabinose oxidation by NADP^+ were analysed after incubation (in a volume of 2.0 ml) of cell extract (3.8 mg protein) with 80 μ mol Tris buffer (pH 8.5), 20 μ mol L-arabinose, 4 μ mol NADP^+ and 1 μ mol EDTA, and the addition of 330 μ mol HCl after 30 min to stop the reaction. The lactone content was the same whether or not the supernatant was heated with HCl before analysis. After rotary evaporation to dryness at 30 °C, the mixture was dissolved in 0.2 ml water, treated with 0.1 g moist Dowex-50W (100–200 mesh, H^+ form), centrifuged, and the supernatant used for paper chromatography.

The products from arabinono- γ -lactone were followed after incubating (in a volume of 2.5 ml) cell extract (3.8 mg protein), 20 μ mol arabinono- γ -lactone and 125 μ mol HEPES. After addition of 330 μ mol HCl to halt the reaction, the supernatant was treated with 0.1 g Dowex-50W as above and rotary evaporated at 30 °C to 0.2 ml and re-centrifuged before chromatography.

L-Arabinonate was incubated in the standard assay system for arabinonate dehydratase for 43 min, then 0.5 ml 25 mM-2,4-dinitrophenylhydrazine in 2 M-HCl was added. After 20 min incubation at 30 °C, the mixture was stored frozen before extraction of the 2,4-dinitrophenylhydrazones into ethyl acetate for chromatography.

The products from KDA were generated by incubating (in a final volume of 1.0 ml) cell extract (1 mg protein), 100 μ mol potassium phosphate (pH 7.6), 2 μ mol 2-mercaptoethanol and 7 μ mol DL-KDA for 20 min at 30 °C. To one such incubation was added 5 ml 5 mM-2,4-dinitrophenylhydrazine in 2 M-HCl, while 2 μ mol NAD^+ was added to the other, followed by a further 20 min incubation at 30 °C before a similar amount of 2,4-dinitrophenylhydrazine was added. Hydrazone formation in both cases was allowed to proceed for 15 min at 30 °C and overnight at 4 °C. Dinitrophenylhydrazones were then extracted from the aqueous layers into ethyl acetate for paper chromatography.

Chromatography. All separations were carried out by descending chromatography on Whatman no. 1 paper at 18–25 °C. 2,4-Dinitrophenylhydrazones of oxo-acids were separated using 1% (w/v) aqueous Na_2CO_3 (Hockenhull *et al.*, 1953), 0.1 M-glycine buffer (pH 8.5) (Virtanen *et al.*, 1953), butan-1-ol/ethanol/water (70:10:20, by vol.; Cavallini *et al.*, 1949) or propan-2-ol/water/ammonia (200:20:10, by vol.; Smith, 1960). Spots were identified by UV absorbance and by colour reaction with alcoholic NaOH (Smith, 1960).

L-Arabinose, mannitol, L-arabinono- γ -lactone, L-arabinonate, KDA and 2-oxoglutarate were separated using propanol/formic acid/water (6:3:1, by vol.; Weimberg, 1959), ethyl acetate/pyridine/water (12:5:4, by vol.; Smith, 1960) and butan-2-ol (purified as described by Weimberg, 1959)/formic acid (95:5, v/v). All were detectable with alcoholic AgNO_3 followed by alcoholic NaOH. Lactones were identified by reagents modified from Abdel-Akher & Smith (1951). Equal volumes of 1 M- NH_2OH , HCl and 1.1 M-KOH in methanol were mixed, filtered and used as the first dipping solution, followed by 1% (w/v) FeCl_3 in methanol containing 3 ml conc. HCl per 100 ml. Sugars were identified with an aniline/diphenylamine/ H_3PO_4 reagent (Smith, 1960). Oxo-acids were detected with 0.5% (w/v) alcoholic 2,4-dinitrophenylhydrazine, followed by 2% (w/v) NaOH (Smith, 1960).

Analytical. L-Arabinono- γ -lactone was determined by the method of Hestrin (1949); L-arabinonate was first converted to the γ -lactone. DL-KDA was estimated after periodate oxidation for 20 min at 30 °C under the conditions used by Weissbach & Hurwitz (1959), or as the semicarbazone (MacGee & Doudoroff, 1954). 2-Oxoglutarate semialdehyde solutions were standardized as the bis-2,4-dinitrophenylhydrazone in methanol at

433 nm (Dagley & Trudgill, 1965) or as the *bis*-semicarbazone (MacGee & Doudoroff, 1954). 2-Oxoglutarate was determined as the 2,4-dinitrophenylhydrazone, using the assay system for pyruvate (Dilworth, 1962).

Both D-ribose and L-arabinose were determined using the orcinol method of Ashwell (1957).

Protein was measured by the Lowry method with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Route of L-arabinose metabolism in R. leguminosarum

The products of L-arabinose oxidation with NADP^+ in crude extracts of strain MNF300 were identified by chromatography as arabinono- γ -lactone and arabinonate. The γ -lactone gave rise to arabinonate on incubation with cell extract, together with small amounts of an oxo-acid identified as KDA by its R_F and its colour after treatment with alcoholic 2,4-dinitrophenylhydrazine followed by alcoholic NaOH. Incubation of L-arabinonate with crude cell extracts produced an oxo-acid identified from its 2,4-dinitrophenylhydrazones as KDA, and smaller amounts of 2-oxoglutarate. Incubation of DL-KDA with cell extract, followed by chromatography of the 2,4-dinitrophenylhydrazones of the products, showed that 2-oxoglutarate semialdehyde and lesser amounts of 2-oxoglutarate were produced. No pyruvate or glycolaldehyde derivatives were detected. Addition of NAD^+ to mixtures after their incubation with DL-KDA resulted in the disappearance of 2-oxoglutarate semialdehyde and the appearance of larger quantities of 2-oxoglutarate.

Cultures of MNF300 metabolizing L-arabinose accumulated 2-oxoglutarate when treated with 1 mM-sodium arsenite to inhibit the 2-oxoglutarate dehydrogenase; the rate of 2-oxoglutarate accumulation [$4.1 \mu\text{mol h}^{-1} (\text{mg dry weight})^{-1}$] equalled the rate of L-arabinose consumption [$4.3 \mu\text{mol h}^{-1} (\text{mg dry weight})^{-1}$]. Arabinose-metabolizing cells of cowpea *Rhizobium* NGR234 also accumulated stoichiometric amounts of 2-oxoglutarate when exposed to 1 mM-sodium arsenite (data not shown).

Accordingly, it is suggested that in *R. leguminosarum* MNF300 and in cowpea *Rhizobium* NGR234 L-arabinose is metabolized via the 2-oxoglutarate semialdehyde pathway rather than via pyruvate and glycolaldehyde produced by KDA aldolase.

Control of L-arabinose pathway

Activities of the enzymes of the L-arabinose pathway in cells of *R. leguminosarum* MNF300 grown on arabinose, mannitol or succinate (all at 10 mM) are presented in Table 2. Synthesis of L-arabinose dehydrogenase and the lactonase was not tightly controlled; the three later enzymes of the pathway appeared to be more closely regulated and to respond coordinately to growth on mannitol or succinate. The relative activities of the enzymes were consistent with the pattern of products found above; the greater activities of KDA dehydratase and 2-oxoglutarate semialdehyde dehydrogenase restrict KDA accumulation from L-arabinonate. Growth of MNF300 on 10 mM-mannitol plus 10 mM-arabinose did not result in catabolite repression of the arabinose enzymes (Table 2).

KDA aldolase activity as measured was very low; assay of this activity as KDA synthesis is impractical in the face of a relatively active KDA dehydratase. The major indications that the aldolase is unimportant come from the facts that incubation of DL-KDA with cell-free extracts gave rise to neither pyruvate nor glycolaldehyde, and that treatment of arabinose-metabolizing whole cells with arsenite gave rise to 2-oxoglutarate in stoichiometric amounts.

The activities of the enzymes of L-arabinose metabolism in pea bacteroids are also presented in Table 2; arabinose dehydrogenase and arabinono- γ -lactonase were readily detected, while arabinonate dehydratase, KDA dehydratase and KDA aldolase were at very low activity or absent. 2-Oxoglutarate semialdehyde dehydrogenase activity was present at high levels. The significance of this result is not clear. It suggests either that this enzyme can be regulated independently of the preceding two and may be associated with catabolism of other compounds such as hydroxyproline, glucarate or galactarate (Dagley & Trudgill, 1965), or that the activity is

Table 2. Activities of arabinose catabolic enzymes [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] in cell-free extracts of *R. leguminosarum* MNF300 grown on 10 mM-arabinose, mannitol or succinate, or 10 mM-arabinose plus 10 mM-mannitol

Strain	Growth substrate	Except where indicated, values are the mean of at least two assays, and are shown $\pm$ the standard deviation.				
		Arabinose dehydrogenase	Arabinono- γ -lactonase	Arabinonate dehydratase	KDA dehydratase	KDA aldolase
MNF300	Arabinose	101 $\pm$ 16	176 $\pm$ 63	36 $\pm$ 2	50 $\pm$ 6	4*
	Mannitol	60 $\pm$ 11	177 $\pm$ 24	9 $\pm$ 3	15 $\pm$ 13	2*
	Succinate	43 $\pm$ 4	95 $\pm$ 2	0	3 $\pm$ 1	ND
MNF3045	Mannitol + arabinose	156 $\pm$ 39	142 $\pm$ 24	30 $\pm$ 5	88 $\pm$ 37	ND
	Mannitol + arabinose	87 $\pm$ 28	91 $\pm$ 26	0	3 $\pm$ 3	ND
MNF300	Bacteroids	53 $\pm$ 8	109 $\pm$ 30	1 $\pm$ 2	0	0.4*
2-Oxoglutarate semialdehyde dehydrogenase						
						115 $\pm$ 35
						39 $\pm$ 13
						18 $\pm$ 3
						166 $\pm$ 13
						3 $\pm$ 5
						96 $\pm$ 8

ND, Not determined. * One assay only.

Table 3. Activities of arabinose catabolic enzymes [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] in cell-free extracts of cowpea *Rhizobium* NGR234 grown on 10 mM-arabinose, glucose or succinateValues are the mean of at least two assays, $\pm$ the standard deviation.

Growth substrate	Arabinose dehydrogenase	Arabinono- γ -lactonase	Arabinonate dehydratase	KDA dehydratase	KDA aldolase	2-Oxoglutarate semialdehyde dehydrogenase
Arabinose	95 $\pm$ 17	92 $\pm$ 15	54 $\pm$ 23	36 $\pm$ 4	0.3 $\pm$ 0.1	204 $\pm$ 25
Glucose	6 $\pm$ 1	79 $\pm$ 32	0	9 $\pm$ 9	3 $\pm$ 2	18 $\pm$ 53
Succinate	10 $\pm$ 4	74 $\pm$ 35	0	17 $\pm$ 8	3 $\pm$ 2	17 $\pm$ 4
Bacteroids	19 $\pm$ 6	49 $\pm$ 27	2 $\pm$ 3	0	0.5 $\pm$ 0.5	5 $\pm$ 1

due to a non-specific aldehyde dehydrogenase similar to that recorded by Peterson & LaRue (1982) for bacteroids of *B. japonicum*.

In cowpea *Rhizobium* NGR234, arabinose dehydrogenase, arabinonate dehydratase, KDA dehydratase and 2-oxoglutarate semialdehyde dehydrogenase were essentially inducible by arabinose, while only the arabinono- γ -lactonase was present at significant activities in cells grown on glucose or succinate (Table 3). Since snake bean bacteroids show only low activities of arabinose dehydrogenase and no activity for arabinonate dehydratase and KDA dehydratase, it seems unlikely that L-arabinose is supplied to these bacteroids.

Incubation of bacteroids of *R. leguminosarum* MNF300 or of cowpea *Rhizobium* NGR234 with L-arabinose in the presence of 1 mM-sodium arsenite resulted in only a very slow accumulation of 2-oxoglutarate [$1\text{--}2 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], suggesting that the L-arabinose pathway is not operative in either type of bacteroid.

Properties of the Tn5-induced arabinose-negative (Ara⁻) mutant MNF3045

MNF3045, derived from MNF300 by Tn5 mutagenesis, was unable to grow on L-arabinose as sole carbon source. Linkage of Tn5 to the Ara⁻ determinant of this strain was demonstrated by their cotransduction by phage RL38. The Ara⁻ phenotype of MNF3045 reverted to Ara⁺ at frequencies of $10^{-6}\text{--}10^{-5}$. Revertants of MNF3045 were analysed: each of 20 revertants tested had lost the kanamycin-resistance marker of Tn5. Thus, Tn5 appears to excise precisely at high frequency from its site of insertion in MNF3045. The reversion of Tn5-induced mutations, at high frequency, has not previously been observed in derivatives of *R. leguminosarum* MNF300 (Glenn *et al.*, 1984a, b) and appears to be rare in other bacteria (e.g. *Erwinia*: Chatterjee *et al.*, 1983). In *Escherichia coli*, the frequency of precise excision of Tn5 is known to be dependent on the site of insertion; frequencies as high as $10^{-6}\text{--}10^{-5}$ have been found only at certain sites in F'::Tn5 plasmids (Berg *et al.*, 1983; Egner & Berg, 1981).

Arabinose catabolic enzymes in Ara⁻ mutants

Activities of arabinose catabolic enzymes in extracts from MNF3045 grown on 10 mM-mannitol plus 10 mM-L-arabinose are compared with those for MNF300 grown on the same substrates in Table 2. The levels of arabinose dehydrogenase and arabinono- γ -lactonase were similar to those found in extracts of arabinose-grown MNF300. MNF3045 did not produce measurable levels of arabinonate dehydratase; the levels of KDA dehydratase and 2-oxoglutarate semialdehyde dehydrogenase were also low. The data strongly suggest that the last three enzymes of the pathway are regulated quite independently of the first two. Whether the mutation of MNF3045 occurs in a control gene or as a polar mutation in a polycistronic operon remains to be determined.

MNF3400 is a spontaneous mutant of MNF300 which has gained the ability to transport L-arabinonate rapidly [$18\text{--}30 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$], and to grow on it with a doubling time of 3.3 h. By contrast, MNF300 transported L-arabinonate at $2\text{--}3 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ and failed to grow on it.

When the mutation in MNF3045 was transduced into MNF3400, the resulting strain (MNF340045) was unable to grow on either L-arabinose or L-arabinonate. Since cells of

Table 4. Concentrations of compounds in culture supernatants of MNF3045 and MNF340045 grown on 10 mM-mannitol plus 10 mM-L-arabinose

Mutant	Concn (mM) of:		Change in arabinose concn (mM)
	Arabinono- γ -lactone	Arabinonate	
MNF3045	2.0	4.0	6.2
MNF340045	0.3	3.5	3.5

Table 5. Activities of ribose transport and catabolic enzymes [$\text{nmol min}^{-1} (\text{mg protein})^{-1}$] in *R. leguminosarum* strains MNF300 and MNF3041 grown on 10 mM-glucose plus 10 mM-ribose

Values are the mean of at least two assays, $\pm$ the standard deviation.

Strain	Ribose transport rate	Ribokinase	Ribose-5-phosphate isomerase	Ribulose-5-phosphate epimerase	Transketolase
MNF300	16 ± 8	18 ± 1	35 ± 6	64 ± 20	68 ± 14
MNF3041	18 ± 2	0	35 ± 15	87 ± 4	44 ± 11

MNF3400 can take up and grow on L-arabinonate, these results confirm that the lesion in MNF3045 must block arabinose catabolism at a step subsequent to L-arabinonate.

Accumulating compounds in cultures of MNF3045 and MNF340045

Mutants of *R. meliloti* unable to grow on L-arabinose can nevertheless metabolize it (Duncan, 1981). When *R. leguminosarum* MNF3045 or MNF340045 was grown on 10 mM-mannitol plus 10 mM-L-arabinose for 24 h, much of the L-arabinose disappeared (Table 4). The supernatant contained a compound reactive with alkaline hydroxylamine and another compound which could be converted to a reactive one by treatment in 0.2 M-HCl for 5 min at 100 °C. Chromatography of the material from MNF3045 in the ethyl acetate/pyridine/water solvent, which resolved mannitol, L-arabinose, L-arabinonate and L-arabinono- γ -lactone, showed that all four compounds were present in the culture filtrate. Cultures of MNF300 grown under similar conditions did not accumulate either L-arabinonate or L-arabinono- γ -lactone but a low concentration of 2-oxoglutarate (0.25 mM) was detected.

The amounts of L-arabinose metabolized by mutants MNF3045 and MNF340045 corresponded to the total of L-arabinonate plus L-arabinono- γ -lactone accumulated in the medium (Table 4). The excretion of L-arabinonate and L-arabinono- γ -lactone indicates that L-arabinonate cannot be further metabolized.

The mutant of *R. meliloti* described by Duncan (1981) as being able to metabolize L-arabinose but not to grow on it may well behave like *R. leguminosarum* MNF3045 and MNF340045 and accumulate intermediates in a similar way.

Properties of the ribose-negative (Rib⁻) mutant MNF3041

Mutant MNF3041 was unable to grow on ribose as sole source of carbon. When incubated in 5 mM-ribose following growth on 5 mM-glucose plus 5 mM-ribose, it degraded ribose at a rate of $0.54 \mu\text{mol h}^{-1} (\text{mg dry weight})^{-1}$; MNF300 under similar conditions grew with a generation time of 6.7 h and catabolized ribose at a rate of $2.02 \mu\text{mol h}^{-1} (\text{mg dry weight})^{-1}$. The consumption of ribose by MNF3041 also resulted in incorporation of radioactivity from [^{14}C]ribose into ethanol-insoluble cell material (data not shown); some 74% of the total radioactivity taken up was found in the insoluble fraction. Thus, MNF3041 appears to be similar in phenotype to the Rib⁻ mutant MD5 of *R. meliloti* (Duncan, 1981) in its failure to grow on ribose but its ability to utilize it.

The lesion in MNF3041 appears most likely to be an inability to produce ribokinase, since cells of MNF3041 take up ribose at rates at least as high as those for MNF300 (Table 5) and

other early enzymes of ribose catabolism via the oxidative pentose phosphate pathway are present in extracts of MNF3041 grown on glucose plus ribose. This conclusion is consistent with the ability of MNF3041 to grow on D-ribitol (a precursor of D-ribulose) or D-arabitol (a precursor of D-xylulose).

As with other mutants of *R. leguminosarum* which cannot use C₆ or C₁₂ sugars for growth (Glenn *et al.*, 1984*a, b*) and with mutants of *R. meliloti* unable to use pentoses, mutants of *R. leguminosarum* unable to use L-arabinose or D-ribose are still able to form nodules and to fix N₂ normally. No absolute requirement for large exogenous supplies of either of these compounds can therefore be necessary for nodule formation or function.

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